# Enhanced Thalamic Spillover Inhibition during Non–rapid-eye-movement Sleep Triggers an Electrocortical Signature of Anesthetic Hypnosis

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## ABSTRACT

**Background:** Alterations in thalamic γ-aminobutyric acid–mediated signaling are thought to underlie the increased frontal α-β frequency electrocortical activity that signals anesthetic-induced loss of consciousness with γ-aminobutyric acid receptor type A ($GABA_AR$)–targeting general anesthetics. The general anesthetic etomidate elicits phasic extrasynaptic $GABA_AR$ activation ("spillover" inhibition) at thalamocortical neurons *in vitro*. We hypothesize that this action of etomidate at the thalamus is sufficient to trigger an increase in frontal α-β frequency electrocortical activity and that this effect of etomidate is fully recapitulated by enhanced thalamic spillover inhibition *in vivo*.

**Methods:** We recorded electrocortical activity and sleep–wake behavior in freely behaving wild-type (n = 33) and extrasynaptic δ-subunit–containing $GABA_AR$ knockout mice (n = 9) during bilateral microperfusion of the thalamus with etomidate and/or other pharmacologic agents that influence $GABA_AR$ or T-type $Ca^{2+}$ channel activity.

**Results:** Microperfusion of etomidate into the thalamus elicited an increase in α-β frequency electrocortical activity that occurred only during non–rapid-eye-movement (REM) sleep (11.0 ± 11.8% and 16.0 ± 14.2% greater 8 to 12- and 12 to 30-Hz power, respectively; mean ± SD; both $P < 0.031$) and was not affected by blockade of thalamic T-type $Ca^{2+}$ channels. Etomidate at the thalamus also increased spindle-like oscillations during non-REM sleep (4.5 ± 2.4 spindle per minute with etomidate *vs.* 3.2 ± 1.7 at baseline; $P = 0.002$). These effects of etomidate were *fully* recapitulated by enhanced thalamic extrasynaptic $GABA_AR$-mediated spillover inhibition.

**Conclusions:** These findings identify how a prototypic $GABA_AR$-targeting general anesthetic agent can elicit the characteristic brain wave pattern associated with anesthetic hypnosis when acting at the thalamus by promoting spillover inhibition and the necessity of a preexisting non-REM mode of activity in the thalamus to generate this effect. **(ANESTHESIOLOGY 2016; 125:964-78)**

THE electrocortical patterns that signal anesthetic-induced loss of consciousness are only starting to be understood.[1] There is an established link between increased frontal α-β frequency cortical activity and the onset of the behavioral correlates that mark loss of consciousness with γ-aminobutyric acid receptor type A ($GABA_AR$)–targeting general anesthetic agents.[2–4] Moreover, a critical role for the thalamus has been implicated in orchestrating the emergence of this α-β frequency electrocortical signature during the induction of anesthesia.[1,3–5] Specifically, *in vivo* local field potential recordings in rodents identify that during propofol-induced loss of consciousness, elevated α-β activity in the thalamus precedes manifestation of the same signature in the cortex.[4] Additionally, computational modeling indicates that increased γ-aminobutyric acid–mediated (GABAergic) conductance and prolonged inhibitory postsynaptic potentials (IPSPs) in thalamocortical neurons can reinforce cortical activity in the α-β frequency range.[3,5] Importantly, the molecular and cellular underpinnings that could position the thalamus to orchestrate these electrocortical changes remain to be elucidated *in vivo*.

### What We Already Know about This Topic

- The administration of anesthetics that target the γ-aminobutyric acid receptor type A leads to increased frontal α-β frequency activity on the electroencephalogram during loss of consciousness.
- The increased α-β activity in the cortex is preceded by a similar increase in α-β activity in the thalamus. The means by which thalamic activity coordinates cortical α-β activity in the cortex are not clear.
- Microperfusion of the thalamus in rodents was employed to determine the impact of the γ-aminobutyric acid–mediated anesthetic etomidate on thalamic activity and its role in the cortical electroencephalogram signature of hypnosis.

### What This Article Tells Us That Is New

- Microperfusion of etomidate increased non–rapid-eye-movement α-β electrocortical activity and decreased wakefulness.
- The thalamus is implicated in triggering this increase in α-β frequency activity in the cortex during anesthetic hypnosis. The means by which thalamic activity coordinates cortical α-β activity in the cortex are not clear.
- The data suggest that the sedative properties of etomidate first lead to a non–rapid-eye-movement sleep state, followed by a spillover extrasynaptic γ-aminobutyric acid receptor type A–mediated inhibition in the thalamus, which then leads to thalamocortical oscillation in the α-β range.

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Modulation of thalamic GABAergic signaling can trigger state-associated changes in electrocortical activity.[6] There are three types of $GABA_A$R-mediated inhibition identified in thalamocortical neurons: phasic (*i.e.*, phasic activation of synaptic $GABA_A$Rs), tonic (*i.e.*, tonic activation of extra- and perisynaptic $GABA_A$Rs), and spillover (*i.e.*, phasic activation of extra- and perisynaptic $GABA_A$Rs).[7] Importantly, the alterations in thalamocortical neuron firing elicited by the general anesthetic etomidate require phasic activation of extrasynaptic $GABA_A$Rs *in vitro*.[8]

T-type $Ca^{2+}$ channel activation plays a critical role in mediating slow, 1 to 4-Hz oscillatory signaling in thalamocortical neurons.[6] These channels become activated at hyperpolarized resting membrane potentials, *i.e.*, membrane potentials lower than approximately −65 mV. Such hyperpolarized resting membrane potentials occur naturally in thalamocortical neurons during non–rapid-eye-movement (REM) sleep when GABAergic signaling from the reticular thalamic nucleus (RTN) is elevated and depolarizing inputs from ascending arousal nuclei are attenuated. Moreover, thalamic T-type $Ca^{2+}$ channel activity is associated with increased 1 to 4-Hz electrocortical activity, which is considered a hallmark of deep non-REM sleep.[6,9]

Here we use a combination of genetic, pharmacologic, and electrophysiologic approaches to identify the electrocortical effects elicited by pharmacologic enhancement of phasic extrasynaptic $GABA_A$R activity in the thalamus *in vivo*. We also identify that the *same* electrocortical signature is produced by the presence of the prototypic $GABA_A$R-targeting general anesthetic agent etomidate at the thalamus *in vivo*. We further characterize the role of thalamic T-type $Ca^{2+}$ channels in mediating these changes in electrocortical activity and identify that this signature is confined to alterations in thalamic activity during non-REM sleep only. Together, these findings identify how a commonly used general anesthetic agent when acting at the thalamus can elicit the characteristic brain wave pattern associated with anesthetic hypnosis and the necessity of an initial sleep transition to generate this signature.

## Materials and Methods

### Animal Care

Three to 6-month-old male wild-type mice (C57BL/6 × SvJ129; n = 33) and *Gabrd*$^{-/-}$ mice (*i.e.*, mice that lack $\delta GABA_A$Rs; n = 9) were used for all experiments (fig. 1A). Mice were generated and housed as previously described.[10,11] All experimental procedures were approved by the University of Toronto Animal Care Committee and performed in compliance with the requirements of the Canadian Council on Animal Care.

### Experimental Protocol

All surgeries were performed under sterile conditions using isoflurane anesthesia (1.5 to 2%). The mice were implanted with frontal electroencephalogram and nuchal electromyogram electrodes for recording of frontal electrocortical activity and sleep–wake behavior, and two microdialysis guide cannulae (CXG-4; Eicom, USA) were stereotaxically positioned 3.0 mm above the right and left ventrobasal complex of the thalamus, *i.e.*, bilateral placement (1.6 mm posterior to lambda, 2 mm lateral to midline, and lowered 0.25 mm ventrally; fig. 1, B to D).[12] After recovery from surgery, mice were habituated to the recording chamber as previously described.[10]

The drugs used for these studies were (a) etomidate, a general anesthetic agent that acts as a positive allosteric modulator of $GABA_A$Rs; (b) 4-chloro-*N*-[2-(2-thienyl) imidazol[1,2-*a*]pyridin-3-yl]-benzamide (DS2), a positive allosteric modulator of extrasynaptic $\delta GABA_A$Rs; (c) 4,5,6,7-tetrahydroisoxazolo[5,4-*c*]-pyridin-3-ol (THIP), a $\delta GABA_A$R-preferring agonist; and (d) 3,5-dichloro-*N*-[1-(2,2-dimethyl-tetrahydro-pyran-4-ylmethyl)-4-fluoro-piperidin-4-ylmethyl]-benzamide (TTA-P2), a potent T-type $Ca^{2+}$ channel blocker.[13–17] Etomidate, DS2, and THIP were purchased from Tocris Bioscience (United Kingdom), and TTA-P2 was purchased from Alomone Labs (Israel).

Etomidate (30 μM), DS2 (100 μM), and THIP (50 μM) were prepared in artificial cerebrospinal fluid (aCSF) with and without TTA-P2 (300 μM). Importantly, the amount of drug that diffuses across the microdialysis probe membrane into tissue is estimated to be 10 to 18% of the original concentration in the perfusion medium.[18,19] Thus, the effective concentration of etomidate, DS2, and THIP at the ventrobasal complex approximates 3 to 5.4, 10 to 18, and 5 to 9 μM, respectively. Similarly, the effective concentration of TTA-P2 at the thalamus is expected to approximate 30 to 54 μM.

The concentrations of THIP and etomidate were selected based on clinically relevant concentrations. Specifically, human serum and plasma levels of THIP and etomidate at the same effective concentrations as the ones used in this study elicit sedation and anesthetic hypnosis.[20–22] In addition, the concentrations of THIP and etomidate used here are similar to the concentrations used in the *in vitro* studies identifying the effects of THIP and etomidate on tonic, phasic, and spillover $GABA_A$R-mediated inhibition in thalamocortical neurons.[8,23] Similarly, DS2 at concentrations similar to that used here promotes phasic extrasynaptic $\delta GABA_A$R activation in thalamocortical neurons *in vitro*.[7,24] There are no clinically relevant doses with which to compare with DS2 as it does not effectively cross the blood–brain barrier when administered systemically.[14]

The concentration of TTA-P2 was selected based on previous *in vivo* and *in vitro* studies that characterized the specificity of TTA-P2 for low-voltage activated T-type $Ca^{2+}$ channels. Specifically, a previous *in vivo* study, using methods similar to ours, identified that 3 mM TTA-P2 at the ventrobasal complex of naturally sleeping rats significantly reduced thalamocortical burst firing.[25] Importantly, 3 mM

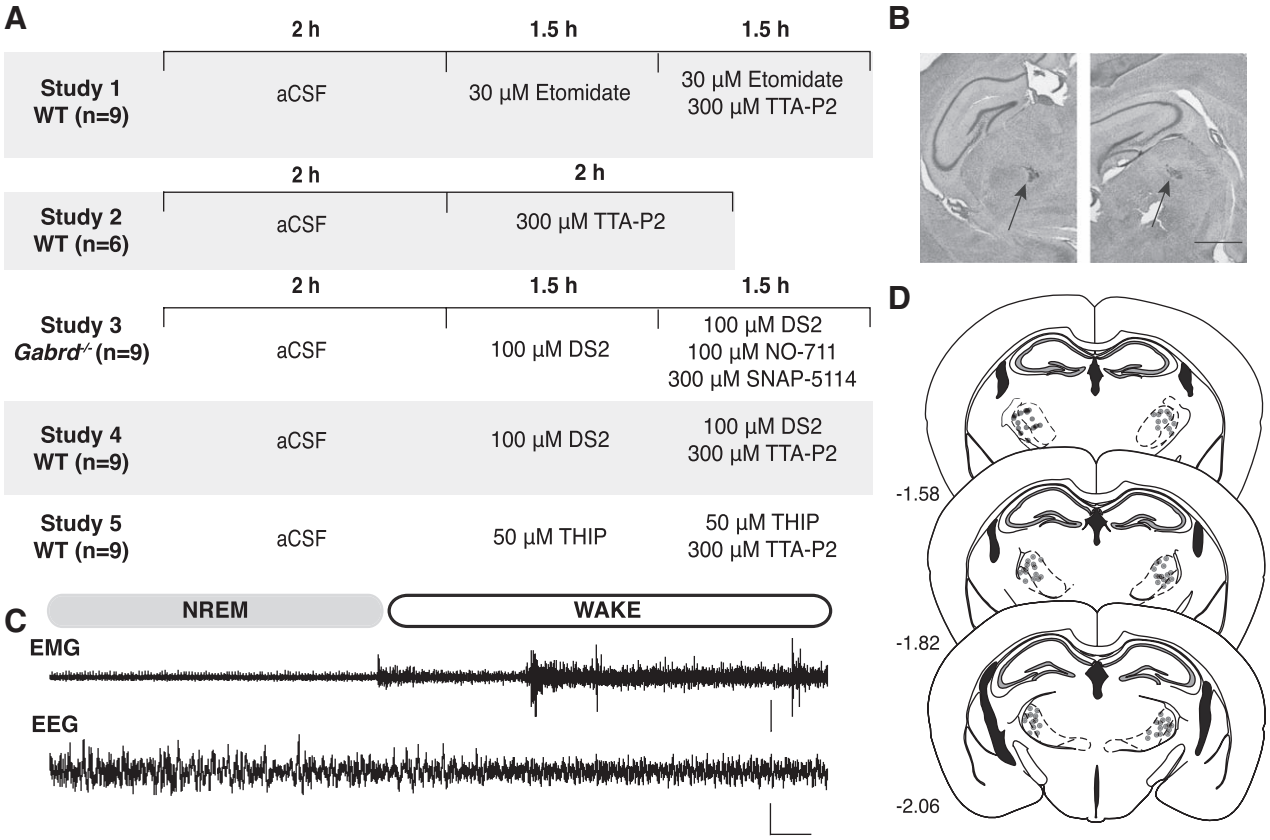


**Fig. 1.** Summary of experimental methods. (*A*) Schema that outlines the treatment protocols for each experimental group. (*B*) Exemplar coronal brain sections showing the targeting of probe tips (as indicated by the *arrows*) to the ventrobasal complex of the thalamus. The *scale bar* equals 1 mm. (*C*) Exemplar raw electroencephalogram (EEG) and electromyogram (EMG) recordings from a mouse during microperfusion of the thalamus with artificial cerebrospinal fluid (aCSF). The *vertical scale bar* below the EMG trace corresponds to the raw EMG recording and equals 400 μV. The *vertical scale bar* below the EEG trace corresponds to the raw EEG recording and equals 200 μV. The *horizontal scale bar* equals 2 s and is common for both the EMG and EEG trace. (*D*) Probe locations for all mice studied (n = 42). Numbers on the bottom left side of each section indicate the distance (in millimeters) posterior from bregma.[12] DS2 = 4-chloro-*N*-[2-(2-thienyl)imidazol[1,2-*a*]pyridin-3-yl]benzamide; *Gabrd*$^{-/-}$ = $GABA_A$ receptor δ-subunit knockout; NO-711 = 1,2,5,6-tetrahydro-1-[2-[[(diphenylmethylene)amino]oxy]ethyl]-3-pyridine-carboxylic acid hydrochloride; NREM = non–rapid-eye-movement sleep; SNAP-5114 = 1-[2-[*tris*(4-methoxyphenyl)methoxy]ethyl]-(*S*)-3-piperidine-carboxylic acid; THIP = 4,5,6,7-tetrahydroisoxazolo[5,4-*c*]pyridin-3-ol; TTA-P2 = 3,5-dichloro-*N*-[1-(2,2-dimethyl-tetrahydro-pyran-4-ylmethyl)-4-fluoro-piperidin-4-ylmethyl]-benzamide; WT = wild-type.

TTA-P2 at the thalamus was also associated with reduced tonic firing, suggesting that at this concentration, TTA-P2 effectively dampened net thalamic activity. This decrease in total thalamic activity suggests that 3 mM TTA-P2 may also be affecting the activity of other channels in addition to T-type $Ca^{2+}$ channels. Consistent with this interpretation, *in vitro* studies have shown that TTA-P2 at concentrations greater than 100 μM can inhibit high-voltage activated $Ca^{2+}$ channels, which are expressed in the RTN and could affect thalamic output.[26,27] Concentrations of TTA-P2 in the low micromolar range, however, are highly selective for all T-type $Ca^{2+}$ channel isoforms.[16,26] Given these findings, we selected a concentration of 300 μM TTA-P2, which is expected to yield an effective concentration of 30 to 54 μM around the microdialysis probe, a concentration that is associated with selectivity for low-voltage activated T-type $Ca^{2+}$ channels.[18,19]

On the morning of experiments, the mice were gently restrained, and microdialysis probes (Eicom) that projected to the ventrobasal complex were inserted. The probes were continuously flushed with aCSF at a flow rate of 2.1 μl/min. The aCSF was composed of (in mM) 125 NaCl, 3 KCl, 1 $KH_2PO_4$, 2 $CaCl_2$, 1 $MgSO_4$, 25 $NaHCO_3$, and 30 D-glucose. aCSF was bubbled with carbon dioxide to a pH of 7.41 ± 0.01. All experiments were performed during the day. The entire experimental protocol occurred over a 5-h period (1,000 to 1,500 h).

Baseline recordings were performed while aCSF was microperfused bilaterally into the ventrobasal complex of each mouse for 2 h (fig. 1, A, B, and D). The perfusion media was then switched to etomidate, TTA-P2, DS2, or THIP. In the first study, 30 μM etomidate was delivered into the thalamus of wild-type mice (n = 9) for 1.5 h, followed by another 1.5-h period where the thalamus was microperfused

with 30 μM etomidate plus 300 μM TTA-P2. A control study was also conducted where aCSF containing only 300 μM TTA-P2 was microperfused into the thalamus of wild-type mice (n = 6) for 2 h after baseline recordings.

The third study also served as a positive control and was designed to confirm the specificity of DS2 for δGABA$_A$Rs. In this study, the ventrobasal complex of the thalamus of *Gabrd*$^{-/-}$ mice (n = 9) was microperfused with 100 μM DS2 for 1.5 h, followed by another 1.5-h period where they received 100 μM DS2 in aCSF that also contained (a) 100 μM 1,2,5,6-tetrahydro-1-[2-[[(diphenylmethylene)amino]oxy]ethyl]-3-pyridine-carboxylic acid hydrochloride (Sigma-Aldrich, USA), an inhibitor of GABA transporter 1, and (b) 300 μM 1-[2-[*tris*(4-methoxyphenyl)methoxy]ethyl]-(*S*)-3-piperidine-carboxylic acid (Tocris Bioscience, United Kingdom), an inhibitor of GABA transporter 3. Reverse microdialysis of 1,2,5,6-tetrahydro-1-[2-[[(diphenylmethylene)amino]oxy]ethyl]-3-pyridine-carboxylic acid hydrochloride and 1-[2-[*tris*(4-methoxyphenyl)methoxy]ethyl]-(*S*)-3-piperidine-carboxylic acid at concentrations similar to the effective concentrations used here is associated with an increase in extracellular GABA concentrations.[28–30] GABA reuptake inhibitors were coapplied as part of this protocol to further increase GABA levels and promote enhanced GABA$_A$R activation with DS2.

In the fourth study, 100 μM DS2 was microperfused into the ventrobasal complex of wild-type mice (n = 9) for 1.5 h, followed by another 1.5-h period where 100 μM DS2 was delivered with 300 μM TTA-P2. The fifth study was similar to the previous one except that 50 μM THIP was microperfused into the thalamus instead of DS2.

At the end of each experiment, the mice were deeply anesthetized with 5% isoflurane and the brains were removed, fixed, and sliced as previously described.[10] Neutral red staining was performed on dried sections to confirm probe site location (fig. 1B).[12]

### Signal Acquisition and Analysis of Sleep–Wake States

Acquisition, filtering, and digitization of the electroencephalogram and electromyogram signals were performed as previously described.[10] Fast Fourier analysis was used to analyze general characteristics of electrocortical activity in each sleep–wake state and was performed as previously described.[10] Briefly, each 5-s epoch of the electroencephalogram was analyzed for absolute power in five bandwidths: 1 to 4, 4 to 8, 8 to 12, 12 to 30, and 10 to 15 Hz. The absolute power in each bandwidth was divided by the total power (1 to 30 Hz) in each epoch, and the normalized powers were then sorted according to the corresponding sleep–wake state. The mean power in each frequency band for each sleep–wake state was then calculated for each treatment in each subject. Epochs that contained movement artifacts were not included in the analyses. Such movement artifacts were identified in 16 of the 42 mice studied but accounted for less than 1% of the total recording time in each mouse.

Sleep–wake states were scored by analyzing electroencephalogram and electromyogram signals in consecutive 5-s epochs using an established automatic scoring protocol.[31] The accuracy of the scoring procedure was confirmed visually and corrected manually as necessary after this visual inspection. The time spent in wakefulness, non-REM sleep, and REM sleep was calculated as a percentage of the total recording time for each treatment in each animal. The incidence and features of spindle-like oscillations were identified using previously described custom-written routines in MATLAB.[10]

### Analysis of Transitions into Non-REM Sleep

Only stable transitions into non-REM sleep were included in this analysis, *i.e.*, transitions where the mouse was awake for more than 30 s and then transitioned into a non-REM sleep episode that also lasted more than 30 s. The wavelet power spectrums, calculated by taking the square of the wavelet transform, were generated using MATLAB routines based on those provided by Torrence and Compo.[32] We used a Morlet Mother wavelet, defined as:

$$\varphi(t) = \pi^{-\frac{1}{4}} e^{i 2\pi f_0} e^{-\frac{t^2}{2}}$$

where $f_0$ is the wavelet central frequency and $t$ is a dimensionless time parameter. The value of $2\pi f_0$ (*i.e.*, $\omega_0$) was set to 6 to satisfy the admissibility criterion, which states that for an integral function, the analyzing wavelet should have a mean of zero.[33] Alterations in wavelet power during microperfusion of the thalamus with DS2, THIP, etomidate, and/or TTA-P2 were identified by following three fixed frequency bands: 1 to 4, 14 to 20, and 20 to 40 Hz. All three of these bands exhibited significant alterations in power that exceeded a 95% confidence level, which was calculated assuming a red-noise background that serves as an effective background spectrum.[32] This procedure was performed because, like electrocortical activity, red-noise background exhibits increasing power for decreasing frequencies.[4,32] The average power in the 1 to 4-, 14 to 20-, and 20 to 40-Hz bands was calculated for each mouse for the 30-s period before and 30-s period after transitions into non-REM sleep.

### Statistical Analysis

Data are expressed as mean ± SD unless otherwise specified. Sample sizes were selected based on previous experience and published data from our laboratory.[10] Mice were randomly assigned to experimental groups, and experimenters were not blinded to genotype. The effects of microperfusion of etomidate, DS2, and THIP into the thalamus, with and without TTA-P2, on electrocortical activity and sleep–wake behavior were assessed using a two-way repeated-measures (RM) ANOVA with the factors being (a) drug treatment and (b) sleep–wake state. The effects of etomidate, DS2, and THIP on spindle-like oscillations were assessed using a one-way RM ANOVA. Finally, comparisons of electrocortical activity during transitions into non-REM sleep were conducted using a two-way RM ANOVA with the factors being (a) drug

treatment and (b) period of transition (*i.e.*, pre- or posttransition into non-REM sleep). Bonferonni-corrected *P* values were used to test statistical significance when *post hoc* comparisons were performed. Differences were considered significant if *P* was less than 0.05 using a two-tailed test. Analyses were performed using SigmaStat software (SPSS, USA).

## Results

### Etomidate at the Thalamus Increases α-β Electrocortical Activity and Spindle-like Oscillations during Non-REM Sleep

We first characterized the electrocortical signature associated with delivery of the prototypic $GABA_A$R-targeting general anesthetic etomidate into the thalamus. This first experiment was performed before the subsequent manipulations of thalamic activity in order to confirm, validate, and replicate the initial finding from our laboratory upon which this new study was based.[10] Bilateral microperfusion of 30 μM etomidate into the ventrobasal complex of freely behaving wild-type mice that were instrumented with frontal electroencephalogram and nuchal electromyogram electrodes (fig. 1, A to D) elicited a sleep state–dependent decrease in 1 to 4-Hz electrocortical activity and a sleep state–dependent increase in 8 to 12- and 12 to 30-Hz electrocortical activities (all $F_{4,26} > 3.17$; $P < 0.031$; fig. 2, A and B), which fully replicates previous findings from our laboratory.[10] Specifically,

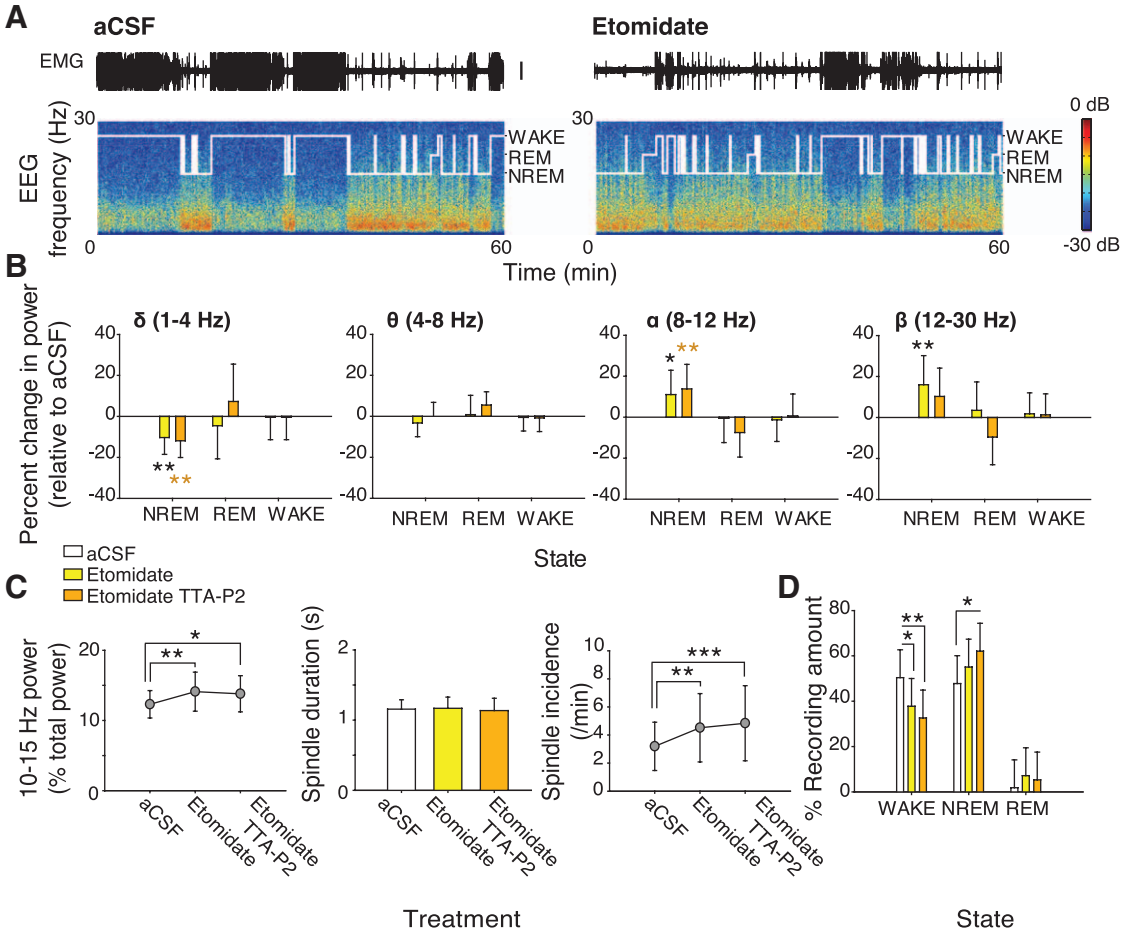


**Fig. 2.** Microperfusion of etomidate into the ventrobasal complex of wild-type mice increases α-β electrocortical activity, sleep spindles, and non–rapid-eye-movement (NREM) sleep through T-type $Ca^{2+}$ channel–independent alterations in thalamocortical activity *in vivo*. (*A*) Exemplar electromyogram (EMG) and electroencephalogram (EEG) recordings from a wild-type mouse during bilateral microperfusion of the ventrobasal complex with artificial cerebrospinal fluid (aCSF; *left*) and etomidate (*right*). The *vertical scale bar* corresponds to the EMG trace and equals 1 mV. Hypnograms are superimposed as white traces on the EEG spectrograms. Note the decrease in slow electrocortical activity with etomidate. (*B*) Microperfusion of etomidate into the thalamus elicits a non-REM sleep–specific reduction in 1 to 4-Hz EEG power and increased 8 to 12-Hz and 12 to 30-Hz powers. These effects persist during coadministration of 3,5-dichloro-*N*-[1-(2,2-dimethyl-tetrahydro-pyran-4-ylmethyl)-4-fluoro-piperidin-4-ylmethyl]-benzamide (TTA-P2) (two-way repeated measures [RM] ANOVA). (*C*) Both NREM 10 to 15-Hz (sigma) power and the incidence of spindle-like oscillations increase with etomidate at the thalamus. Note that coapplication of TTA-P2 with etomidate does not alter these effects and that neither treatment significantly alters the duration of spindle-like oscillations (one-way RM ANOVA). (*D*) Etomidate at the thalamus also elicits a reduction in wakefulness that persists with coapplication of TTA-P2. NREM sleep is increased with etomidate plus TTA-P2 at the thalamus (two-way RM ANOVA). All data represent mean ± SD. n = 9; **P* < 0.05, ***P* < 0.01, and ****P* < 0.001.

etomidate reduced 1 to 4-Hz power and increased both 8 to 12- and 12 to 30-Hz powers, all during non-REM sleep only (*post hoc t* test, all $P < 0.021$). Importantly, mice receiving etomidate at the thalamus spontaneously transitioned in and out of sleep, but the electroencephalogram signature elicited by etomidate occurred only when mice were in non-REM sleep. There was no effect of etomidate at the thalamus on 4 to 8-Hz electrocortical activity ($F_{2, 16} = 2.39$; $P = 0.117$). These initial findings identify that the action of etomidate at the thalamus is sufficient to elicit a cortical activity pattern of anesthetic hypnosis but only when thalamic activity has already entered a mode of firing that is set up during non-REM sleep.

Next we analyzed the effect of etomidate on spindle-like oscillations, given the overlap of α-β frequencies with the frequency of sleep spindles (7 to 14 Hz) that are generated by the thalamus.[6] Etomidate increased 10 to 15-Hz (σ) power during non-REM sleep ($F_{2, 16} = 8.32$; $P = 0.003$; fig. 2C), which further suggests an increase in the density of sleep spindles. We then identified the incidence of individual spindle-like oscillations during non-REM sleep using custom-written routines in MATLAB. Consistent with the alterations in sigma power identified with etomidate at the thalamus, the incidence of spindle-like oscillations was also increased with etomidate ($F_{2, 16} = 15.81$; $P < 0.001$; fig. 2C). Etomidate did not, however, have any effect on the average duration of spindle-like oscillations ($F_{2, 16} = 0.78$; $P = 0.476$; fig. 2C), indicating that once these oscillations were triggered, they proceeded normally. In addition to the alterations in electrocortical activity identified with etomidate at the thalamus, there was also a decrease in the amount of wakefulness ($F_{4, 32} = 4.26$; $P = 0.007$; *post hoc t* test, $P = 0.035$; fig. 2D). Importantly, none of the effects identified above with etomidate at the thalamus (described in paragraphs one and two of the Results section) were affected by coapplication of the T-type $Ca^{2+}$ channel blocker TTA-P2 (300 μM; *post hoc t* test, all $P > 0.555$; fig. 2, B to D). This finding indicates that the changes in electrocortical activity elicited by etomidate at the thalamus were achieved by changes in the thalamocortical activity that was T-type $Ca^{2+}$ channel independent.

We also conducted a separate study to characterize the effects of TTA-P2 at the thalamus alone. This study was important because it showed that microperfusion of TTA-P2 into the thalamus changed electrocortical activity in a manner consistent with reduced slow oscillatory activity in the thalamus, *i.e.*, this study served as a positive control. Specifically, microperfusion of TTA-P2 into the thalamus of wild-type mice significantly altered 1 to 4-, 4 to 8-, 8 to 12-, and 12 to 30-Hz electrocortical activities in a state-dependent manner (all $F_{2, 9} > 4.59$; $P < 0.043$; fig. 3, A and B). Consistent with the role of T-type $Ca^{2+}$ channels in modulating 1 to 4-Hz electrocortical activity, we identified a significant reduction in 1 to 4-Hz power during non-REM sleep with microperfusion of TTA-P2 into the thalamus of wild-type mice (*post hoc t* test, $P = 0.004$).[9] This reduction in 1 to 4-Hz power during non-REM sleep was associated with a concomitant increase in 8 to 12- and 12 to 30-Hz powers (*post hoc t* test, both $P < 0.020$). An effect of TTA-P2 was also identified during REM sleep, with TTA-P2 eliciting an increase in 4 to 8-Hz power and a decrease in 8 to 12- and 12 to 30-Hz powers (all $P < 0.034$; fig. 3B). While 10 to 15-Hz power was increased with TTA-P2 during non-REM sleep ($F_{1, 5} = 10.42$; $P = 0.023$; fig. 3C), there were no corresponding changes in the duration or incidence of spindle-like oscillations (both $F_{1, 5} < 1.42$; $P > 0.287$; fig. 3C). Microperfusion of TTA-P2 into the thalamus did not elicit any alterations in sleep–wake state durations ($F_{1, 5} = 1.11$; $P = 0.340$; fig. 3D).

### Pharmacologically Enhanced Thalamic GABAergic Spillover Inhibition Fully Recapitulates the Effects of Etomidate at the Thalamus

To promote spillover inhibition *in vivo*, we microperfused the ventrobasal complex of freely behaving mice with DS2, which is a positive allosteric modulator of extrasynaptic δ-subunit containing $GABA_A$ ($\delta GABA_A$) receptors.[14] DS2 promotes $\delta GABA_A$R-mediated spillover inhibition in thalamocortical neurons *in vitro* by increasing the sensitivity of extrasynaptic $\delta GABA_A$Rs to GABA released from the synapse and promoting phasic activation of these receptors.[7,14,24]

After having first established that DS2 elicits no identifiable effects on *in vivo* electrocortical activity and sleep–wake state behavior in mice lacking $\delta GABA_A$Rs (*i.e.*, *Gabrd*$^{-/-}$ mice; all $P > 0.08$; fig. 4, A to D), we then performed a similar experiment in wild-type mice. Microperfusion of 100 μM DS2 into the thalamus of freely behaving wild-type mice fully recapitulated the state-dependent alterations in electrocortical activity identified with etomidate at the thalamus (all $F_{4, 26} > 2.76$; $P < 0.044$; fig. 5, A to C). Specifically, DS2 at the thalamus, like etomidate, decreased 1 to 4-Hz power and increased 8 to 12- and 12 to 30-Hz powers during non-REM sleep only (*post hoc* Student's *t* test, all $P < 0.008$; fig. 5B). There was no effect of DS2 on electrocortical activity during wakefulness or REM sleep (*post hoc t* test, all $P > 0.365$; fig. 5B). Microperfusion of DS2 into the thalamus had no effect on 4 to 8-Hz power ($F_{2, 16} = 1.42$; $P = 0.266$; fig. 5B), also consistent with our findings with etomidate. Moreover, like the mice that received etomidate at the thalamus, mice receiving DS2 also spontaneously transitioned in and out of sleep.

Together, these initial findings indicate that using DS2 to increase the sensitivity of extrasynaptic $\delta GABA_A$Rs to phasic GABA release at the thalamus recapitulates the characteristic electrocortical signature elicited by etomidate.[7,14,24] That this signature is confined to non-REM sleep further suggests that there is a state-associated mode of network activity that is necessary for DS2 or etomidate at the thalamus to each elicit this same distinct pattern of electrocortical activity. The increased GABAergic release at thalamic nuclei from the RTN that occurs in non-REM sleep fits with such a mode.[6]

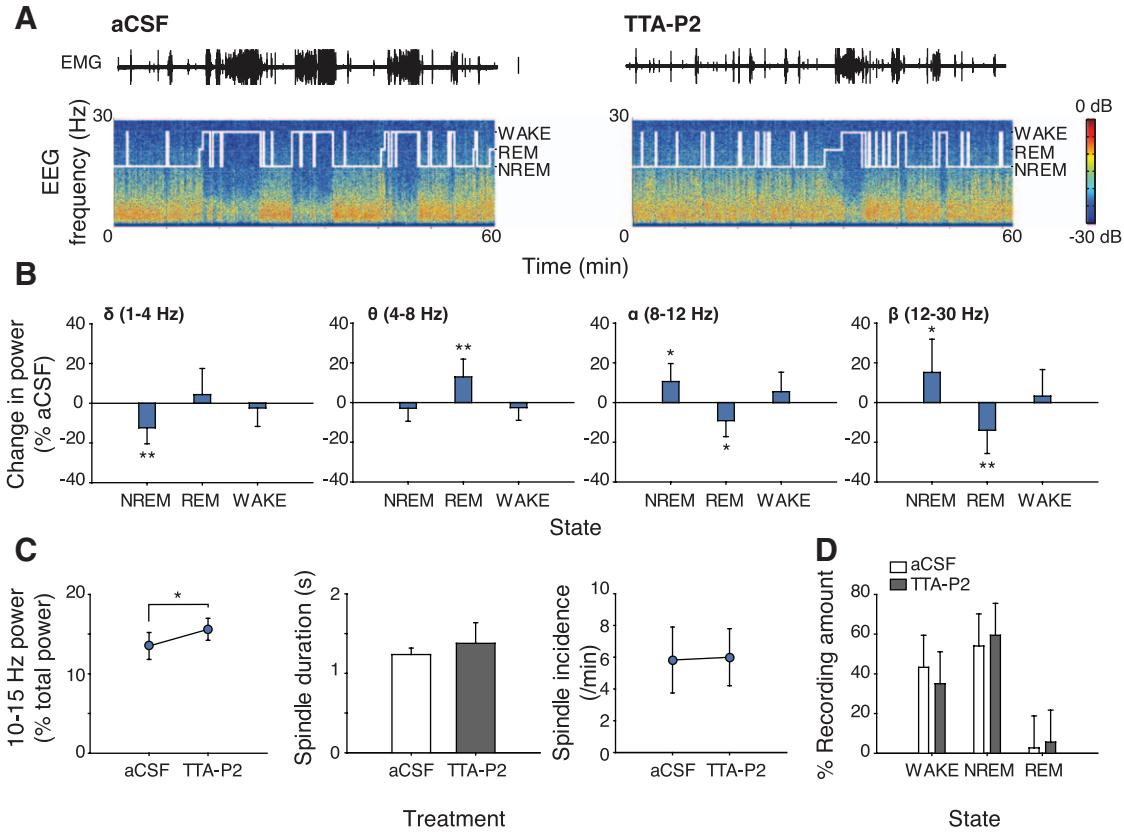


**Fig. 3.** Microperfusion of 3,5-dichloro-*N*-[1-(2,2-dimethyl-tetrahydro-pyran-4-ylmethyl)-4-fluoro-piperidin-4-ylmethyl]-benzamide (TTA-P2) into the thalamus of freely behaving wild-type mice elicits alterations in electrocortical activity consistent with reduced T-type $Ca^{2+}$ channel activity in the thalamus. (*A*) Exemplar electromyogram (EMG) and electroencephalogram (EEG) recordings from a wild-type mouse during microperfusion of the ventrobasal complex with artificial cerebrospinal fluid (aCSF; *left*) and TTA-P2 (*right*). The *vertical scale bar* corresponds to the EMG trace and equals 1 mV. (*B*) During non–rapid-eye-movement (NREM) sleep, blockade of thalamic T-type $Ca^{2+}$ channel activity with TTA-P2 decreased 1 to 4-Hz EEG power and reciprocally increased 8 to 12-Hz and 12 to 30-Hz powers. During REM sleep TTA-P2 at the thalamus increased 4 to 8-Hz EEG power and decreased 8 to 12-Hz and 12 to 30-Hz powers (two-way repeated measures [RM] ANOVA). (*C*) TTA-P2 at the thalamus increased 10 to 15-Hz power during non-REM sleep but had no effect on the duration or incidence of spindle-like oscillations (one-way RM ANOVA). (*D*) Microperfusion of TTA-P2 into the thalamus did not alter sleep–wake state durations (two-way RM ANOVA). All data represent mean ± SD. n = 6; **P* < 0.05 and ***P* < 0.01.

Microperfusion of DS2 into the thalamus also increased 10 to 15-Hz power during non-REM sleep, and this effect occurred with an increase in the incidence of spindle-like oscillations (both $F_{2, 16} > 8.67$; $P < 0.004$) with no accompanying alterations in their average duration ($F_{8, 16} = 2.19$; $P = 0.144$; fig. 5, D to F). The effects of DS2 on spindle-like oscillations again fully mimic the alterations identified with etomidate. DS2 also altered sleep–wake state durations in a manner that paralleled the changes seen with etomidate, with increased non-REM sleep and decreased wakefulness ($F_{4, 32} = 5.41$ $P = 0.002$; *post hoc t* test, both $P < 0.008$; fig. 5G).

The reduction in 1 to 4-Hz electroencephalogram power identified with DS2 (and etomidate) in the thalamus implicates a reduction in slow oscillatory activity in the thalamus. Since thalamic T-type $Ca^{2+}$ channels influence such electrocortical activity, we identified the role of T-type $Ca^{2+}$ channels in mediating the effects of DS2 at the thalamus by coapplying the selective T-type $Ca^{+2}$ channel blocker TTA-P2 into the thalamus of wild-type mice after microperfusion of DS2 alone.[9,34] Coapplication of TTA-P2 with DS2 to the thalamus did not alter any of the electrocortical effects identified with DS2 alone (*post hoc t* test, all $P > 0.344$; fig. 5, B and C). Similarly, there were no differences in spindle-like oscillations between DS2 alone or when it was coapplied with TTA-P2 at the thalamus (*post hoc t* test, both $P > 0.497$; fig. 5, D to F). Coadministration of TTA-P2 with DS2 also did not alter the increased non-REM sleep occurring with DS2 alone (*post hoc t* test, $P = 0.096$), although it did reverse the decrease in wakefulness (*post hoc t* test, DS2 *vs.* DS2 and TTA-P2, $P = 0.026$; aCSF *vs.* DS2 and TTA-P2; $P = 0.361$; fig. 5G). Overall, these findings indicate that thalamic T-type $Ca^{+2}$ channels do not play a significant role in mediating the electrocortical effects elicited by DS2 at the thalamus, again recapitulating the findings with etomidate.

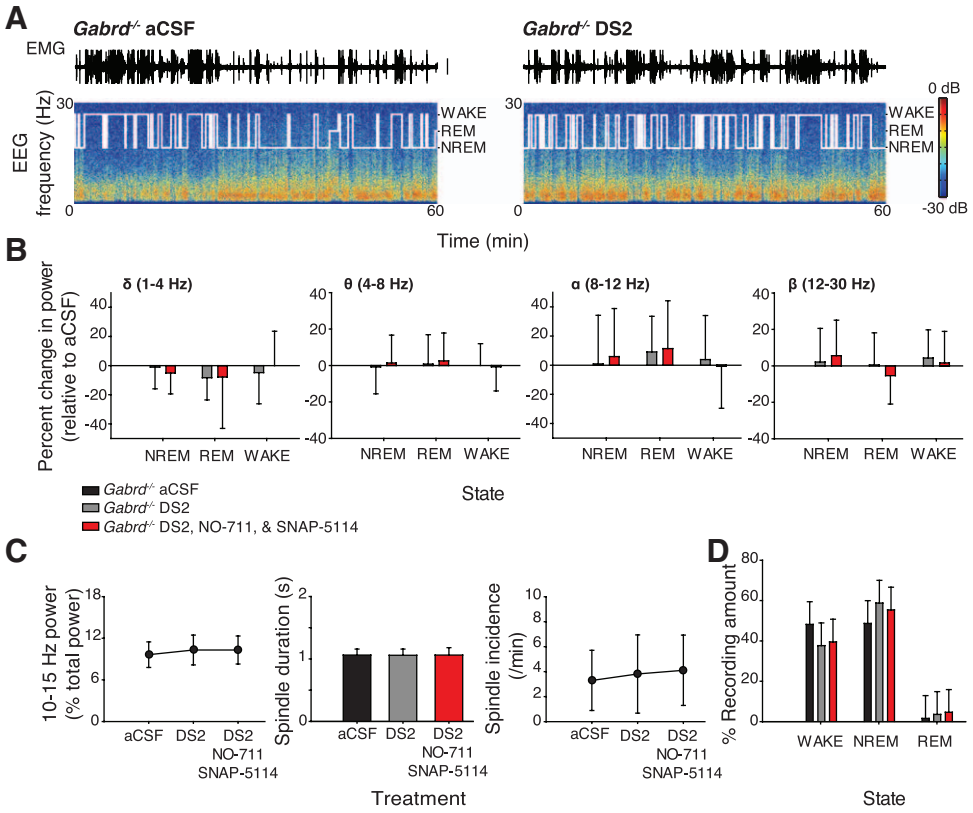


**Fig. 4.** Microperfusion of 4-chloro-*N*-[2-(2-thienyl)imidazol[1,2-*a*]pyridin-3-yl]benzamide (DS2) into the thalamus of freely behaving γ-aminobutyric acid receptor type A ($GABA_A$) receptor δ-subunit knockout (*Gabrd*$^{-/-}$) mice does not alter electrocortical activity or sleep–wake state durations. (*A*) Exemplar electromyogram (EMG) and electroencephalogram (EEG) recordings from a *Gabrd*$^{-/-}$ mouse during microperfusion of the ventrobasal complex with artificial cerebrospinal fluid (aCSF; *left*) and DS2 (*right*). The *vertical scale bar* corresponds to the EMG trace and equals 1 mV. (*B*) DS2 did not elicit any significant alterations in the spectral composition of the EEG during any sleep–wake state in the *Gabrd*$^{-/-}$ mice. Moreover, coadministration of the GABA reuptake blockers 1,2,5,6-tetrahydro-1-[2-[[(diphenylmethylene)amino]oxy]ethyl]-3-pyridine-carboxylic acid hydrochloride (NO-711; a GABA transporter [GAT]-1 blocker) and 1-[2-[*tris*(4-methoxyphenyl)methoxy]ethyl]-(*S*)-3-piperidine-carboxylic acid (SNAP-5114; a GAT-3 blocker) to increase extracellular GABA levels did not elicit an effect of DS2 on electrocortical activity or sleep–wake behavior in the *Gabrd*$^{-/-}$ mice (two-way repeated measures [RM] ANOVA). DS2 did not alter sigma (10 to 15 Hz) power during non–rapid-eye-movement (NREM) sleep or the duration and incidence of spindle-like oscillations in the *Gabrd*$^{-/-}$ mice (one-way RM ANOVA; *C*) nor did it effect sleep–wake state durations (two-way RM ANOVA; *D*). All data represent mean ± SD. n = 9.

### *Pharmacologically Enhanced Thalamic Tonic Inhibition Elicits Alterations in Electrocortical Activity That Are Distinct from Those Identified with Enhanced Thalamic Spillover Inhibition*

To confirm that the effects identified with DS2 were consistent with enhanced phasic extrasynaptic $\delta GABA_AR$ activity (*i.e.*, spillover inhibition) in the thalamus, and not due to elevated tonic $\delta GABA_AR$ activity, we conducted an additional study using THIP to directly activate thalamic $\delta GABA_ARs$ and promote tonic inhibition.[15,35] We have previously shown that microperfusion of THIP into the thalamus of mice that lack $\delta GABA_ARs$ has no effect on electrocortical activity or sleep–wake state durations, confirming the selectivity of THIP for extrasynaptic $\delta GABA_ARs$.[10]

Microperfusion of THIP into the thalamus of wild-type mice elicited an increase in 1 to 4-Hz activity and a decrease in 8 to 12-Hz activity during non-REM sleep and wakefulness (both $F_{2, 16} = 14.06$; $P < 0.001$; *post hoc t* test, both $P < 0.046$; fig. 6, A and B), which replicates previous findings from our laboratory.[10] These effects of THIP were significantly affected by the coapplication of the T-type $Ca^{2+}$ channel blocker TTA-P2 (*post hoc t* test, both $P < 0.037$). TTA-P2 led to a further increase in 1 to 4-Hz power and decreased 8 to 12-Hz power when coapplied with THIP. THIP at the thalamus also elicited state-dependent alterations in 12 to 30-Hz electroencephalogram activity ($F_{2, 16} = 8.30$; $P = 0.003$; fig. 6, A and B), with THIP causing a decrease in 12 to 30-Hz electroencephalogram power during wakefulness (*post hoc t* test, $P = 0.007$). This effect of THIP on 12 to 30-Hz power was also significantly potentiated by coapplication of TTA-P2 (*post hoc t* test; $P = 0.003$). Since only four of nine mice had REM sleep during microperfusion of 50 μM THIP alone and/or in combination with 300 μM TTA-P2 into the thalamus, only the data from non-REM sleep and waking were analyzed for this study.

Microperfusion of THIP into the thalamus also altered spindle-like oscillations, reducing 10 to 15-Hz power

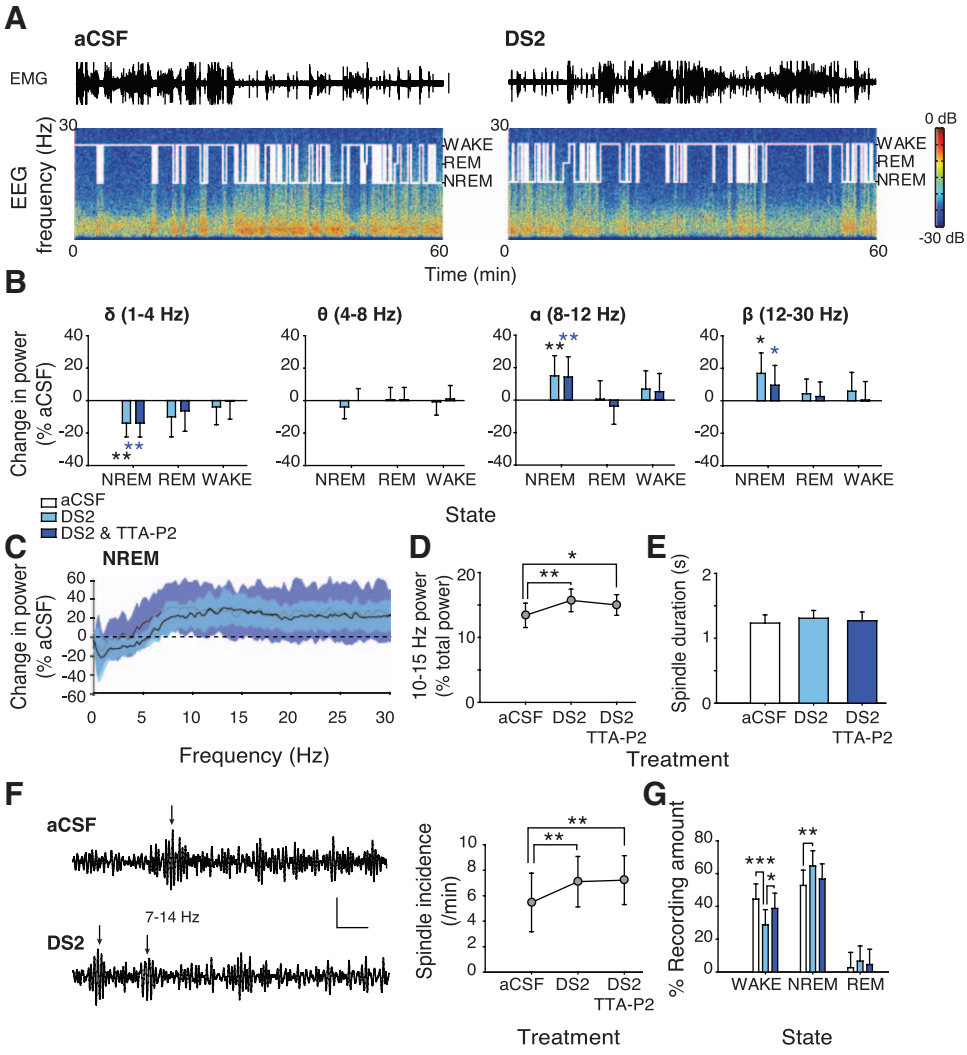


**Fig. 5.** Promoting thalamic spillover inhibition with microperfusion of 4-chloro-*N*-[2-(2-thienyl)imidazol[1,2-*a*]pyridin-3-yl]benzamide (DS2) into the ventrobasal complex of wild-type mice fully recapitulates the alterations in electrocortical activity and sleep–wake state behavior identified with etomidate at the thalamus. (*A*) Exemplar electromyogram (EMG) and electroencephalogram (EEG) recordings from a wild-type mouse as it received artificial cerebrospinal fluid (aCSF; *left*) and DS2 (*right*) bilaterally into the ventrobasal complex. The *vertical scale bar* corresponds to the EMG trace and equals 1 mV. Note the reduced power at low frequencies with DS2. (*B*) DS2 at the thalamus was associated with a non–rapid-eye-movement (NREM) sleep-specific reduction in 1 to 4-Hz EEG power and increased 8 to 12- and 12 to 30-Hz powers. These effects of DS2 were not altered by coapplication of the T-type $Ca^{2+}$ channel blocker 3,5-dichloro-*N*-[1-(2,2-dimethyl-tetrahydro-pyran-4-ylmethyl)-4-fluoro-piperidin-4-ylmethyl]-benzamide (TTA-P2) (two-way repeated measures [RM] ANOVA). (*C*) Continuous power plot showing the decrease in slow frequency electrocortical activity and increase in high-frequency activity with DS2 and DS2 plus TTA-P2. The *central lines* denote the mean change in power, and the *shaded regions* correspond to the 95% CI. Note the similarity in the effects on electrocortical activity of DS2 and DS2 plus TTA-P2. (*D*) Sigma power during NREM sleep was increased by DS2, and this effect did not change with coadministration of TTA-P2. (*E*) The average duration of spindle-like oscillations was unaffected by DS2 and DS2 plus TTA-P2. (*F*) Exemplar recordings of spindle-like oscillations during microperfusion of the thalamus with aCSF and DS2 (*left*). The *vertical scale bar* equals 100 μV, and the *horizontal scale bar* equals 1 s. The incidence of spindle-like oscillations increased with DS2, and this increase was not influenced by TTA-P2 (*right*) (one-way RM ANOVA). (*G*) DS2 at the thalamus also increased the amount of NREM sleep and decreased wakefulness, an effect that was partly reversed by coadministration with TTA-P2 (two-way RM ANOVA). Unless otherwise specified, all data represent mean ± SD. n = 9; **P* < 0.05, ***P* < 0.01, and ****P* < 0.001.

during non-REM sleep ($F_{2,16}$ = 24.50; $P$ < 0.001; *post hoc* $t$ test, $P$ = 0.003; fig. 6C), an effect that was further potentiated by coadministration of TTA-P2 (*post hoc* $t$ test, $P$ = 0.027). Microperfusion of THIP plus TTA-P2 into the thalamus reduced the duration and incidence of spindle-like oscillations compared to aCSF (both $F_{2,16}$ > 9.51; $P$ < 0.003; *pos hoc* $t$ test, both $P$ = 0.001; fig. 6C). Coapplication of THIP with TTA-P2 into the thalamus also

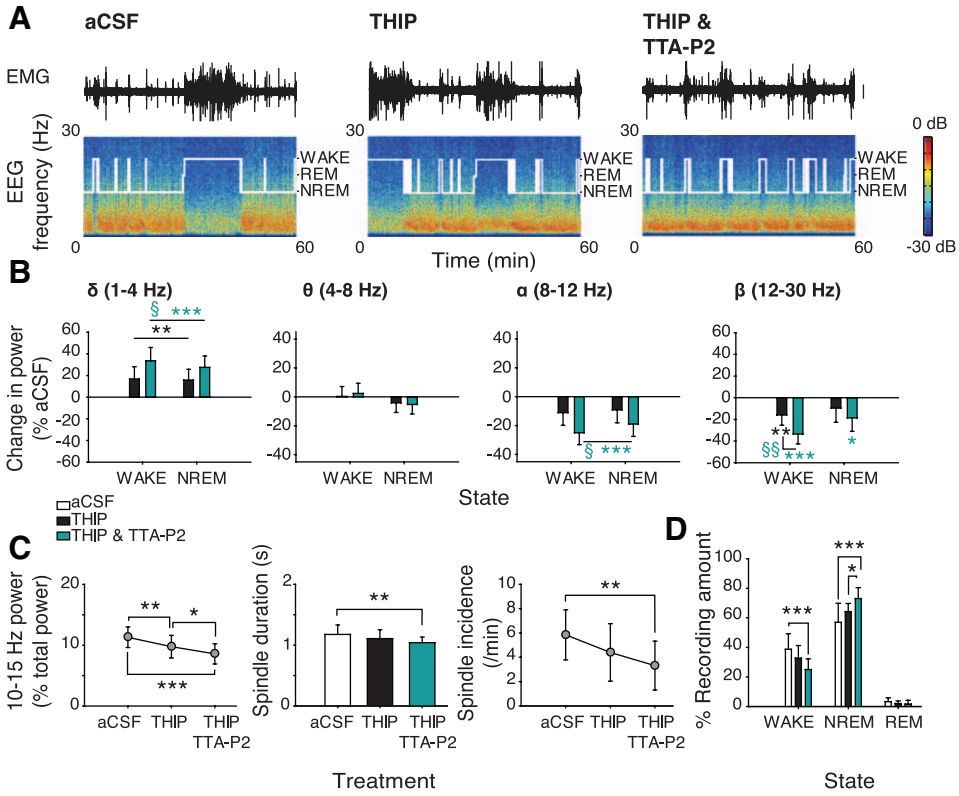


**Fig. 6.** 4,5,6,7-Tetrahydroisoxazolo[5,4-c]pyridin-3-ol (THIP)–induced increases in tonic extrasynaptic γ-aminobutyric acid receptor type A receptor–mediated inhibition in the thalamus elicit distinct alterations in electrocortical activity that differ from 4-chloro-*N*-[2-(2-thienyl)imidazol[1,2-*a*]pyridin-3-yl]-benzamide (DS2) and require T-type $Ca^{2+}$ channel activity *in vivo*. (*A*) Exemplar electromyogram (EMG) and electroencephalogram (EEG) recordings from a mouse during microperfusion of the thalamus with artificial cerebrospinal fluid (aCSF; *left*), THIP (*center*), and THIP with 3,5-dichloro-*N*-[1-(2,2-dimethyl-tetrahydro-pyran-4-ylmethyl)-4-fluoro-piperidin-4-ylmethyl]-benzamide (TTA-P2; *right*). The *vertical scale bar* corresponds to the EMG traces and equals 500 μV. Note the increase in slow electrocortical activity with THIP and the further increase in slow electrocortical activity with THIP and TTA-P2. (*B*) Microperfusion of THIP into the thalamus of wild-type mice elicited a significant increase in 1 to 4-Hz EEG power and a decrease in 8 to 12- and 12 to 30-Hz powers during non–rapid-eye-movement (NREM) sleep and wakefulness. Note that these effects were further increased by coapplication of TTA-P2. The symbol "*" denotes significant differences between baseline (aCSF) and treatment, whereas "§" denote differences between THIP and THIP plus TTA-P2. Rapid-eye-movement (REM) sleep was not included in these comparisons because five mice did not exhibit REM sleep during microperfusion of THIP and/or THIP plus TTA-P2 (two-way repeated measures [RM] ANOVA). (*C*) Microperfusion of THIP into the thalamus decreases non-REM sigma power. Note that microperfusion of TTA-P2 with THIP elicits a further reduction in sigma power and also decreases the duration and incidence of spindle-like oscillations (one-way RM ANOVA). (*D*) THIP at the thalamus does not significantly affect sleep–wake state durations. Note, however, the increase in non-REM sleep and the decrease in wakefulness elicited by TTA-P2 with THIP (two-way RM ANOVA). All data represent mean ± SD. n = 9; § or *$P$ < 0.05, §§ or **$P$ < 0.01, and ***$P$ < 0.001.

altered sleep–wake structure ($F_{4, 32}$ = 7.97; $P$ < 0.001; fig. 6D), with THIP and TTA-P2 eliciting increased non-REM sleep (*post hoc t* test, aCSF *vs.* THIP and TTA-P2, $P$ < 0.001; THIP *vs.* THIP and TTA-P2, $P$ = 0.021) and decreased wakefulness (*post hoc t* test, aCSF *vs.* THIP and TTA-P2, $P$ < 0.001; THIP *vs.* THIP and TTA-P2, $P$ = 0.054).

Importantly, these effects of THIP on electroencephalogram activity and sleep–wake behavior are markedly different from those identified with DS2. This difference indicates that while both agents require δGABA$_A$Rs to elicit their effects, these effects are likely produced by separate mechanisms that can be distinguished through their dependence on thalamic T-type $Ca^{2+}$ channel activity.

### Etomidate and DS2 at the Thalamus Amplify an Electrocortical Signature That Characterizes Entry into Non-REM Sleep

Transient alterations in high-frequency cortical and subcortical activity have been identified during transitions into both non-REM sleep and propofol-induced loss of consciousness in rats.[4] Moreover, this high-frequency signature occurred in the thalamus before the cortex.[4] Accordingly, we then examined transient alterations in electrocortical activity occurring during transitions into non-REM sleep using wavelet analysis. Consistent with the previous report, we identified significant alterations in high-frequency electroencephalogram activity upon entry into non-REM sleep, as indicated by increases in power that exceeded a 95% CI set by a red-noise background (fig. 7, A and B),

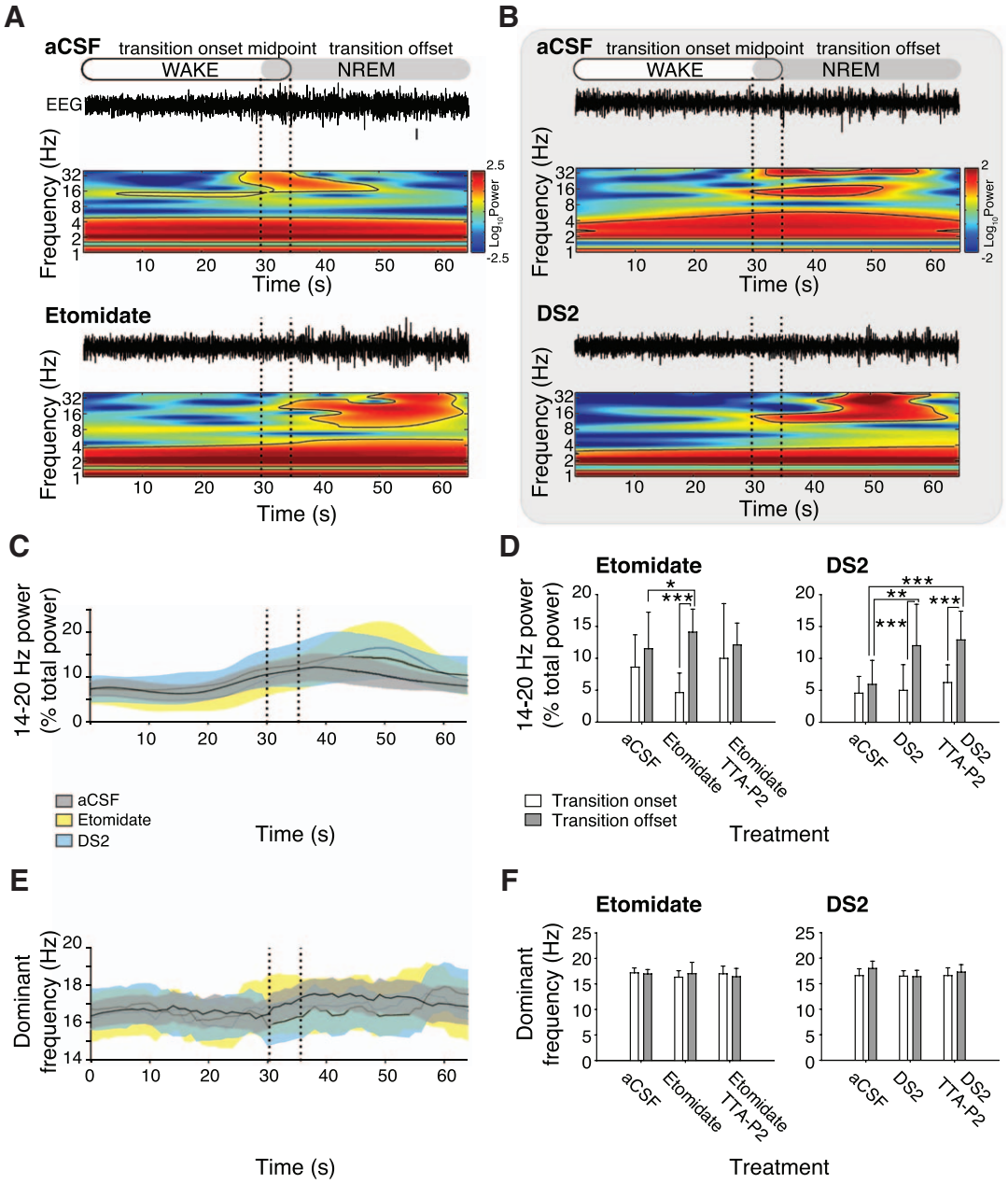


**Fig. 7.** Microperfusion of etomidate or 4-chloro-*N*-[2-(2-thienyl)imidazol[1,2-*a*]pyridin-3-yl]-benzamide (DS2) into the thalamus of wild-type mice amplifies an electrocortical signature characterizing transitions into non–rapid-eye-movement (NREM) sleep. Exemplar electroencephalogram (EEG) recordings from a mouse undergoing a transition into NREM sleep during microperfusion of the thalamus with artificial cerebrospinal fluid (aCSF; *top*) and etomidate (*A*, *bottom*) or DS2 (*B*, *bottom*). The *vertical scale bar* corresponds to the raw EEG traces and equals 100 μV. *Black outlines* on the spectrogram denote spectral regions that show significant alterations in power. Note the increase in power of the same high-frequency EEG signature with both etomidate and DS2. (*C*) Continuous power plot of the 14 to 20-Hz EEG signature of NREM sleep transitions. Note the increase in this power range with etomidate (*yellow*) and DS2 (*blue*) after entry into NREM sleep compared to the aCSF control. The *black lines* denote mean 14 to 20-Hz power for each treatment, and the surrounding *shaded regions* denote the 95% CI. (*D*) Both etomidate (*left*) and DS2 (*right*) increase 14 to 20-Hz power after transitions into NREM sleep. Coadministration of 3,5-dichloro-*N*-[1-(2,2-dimethyl-tetrahydro-pyran-4-ylmethyl)-4-fluoro-piperidin-4-ylmethyl]-benzamide (TTA-P2) with etomidate or DS2 does not significantly affect the increase in 14 to 20-Hz power after entry into NREM sleep (two-way repeated measures [RM] ANOVA). (*E*) Continuous plot of the dominant frequency in the 14 to 20-Hz band during wake–NREM sleep transitions showing the general similarity in the dominant frequency across all treatments. The *black lines* denote mean dominant frequency in the 14 to 20-Hz band for each treatment, and the surrounding *shaded regions* denote the 95% CI. (*F*) The dominant frequency (*i.e.*, frequency at peak power) of the 14 to 20-Hz band is not significantly altered by administration of etomidate (*left*) or DS2 (*right*) into the thalamus with or without coapplication of TTA-P2 (two-way RM ANOVA). Unless otherwise specified, all data represent mean ± SD. n = 9; **P* < 0.05, ***P* < 0.01, and ****P* < 0.001.

the latter also used previously.[4,32] We then identified that microperfusion of etomidate into the thalamus increased the power of the high-frequency (14 to 20 Hz) electroencephalogram signature characterizing entry into non-REM sleep in mice ($F_{2, 12}$ = 5.60; $P$ = 0.019; *post hoc t* test, $P$ = 0.048; fig. 7, C and D; note the period defined as the transition onset at the top of the figure). Microperfusion of etomidate into the thalamus did not alter the power of this electroencephalogram signature during the onset of transitions into non-REM sleep (*post hoc t* test, $P$ = 0.205; fig. 7, C and D). Likewise, microperfusion of DS2 into the thalamus elicited an increase in the 14 to 20-Hz signature after entry into non-REM sleep ($F_{2, 16}$ = 6.64; $P$ = 0.008; *post hoc t* test, $P$ = 0.002) and had no effect on this signature at the onset of transitions (*post hoc t* test, $P$ = 0.778; fig. 7, C and D). The effects of DS2 and etomidate on the 14 to 20-Hz electroencephalogram signature were unaffected by the T-type $Ca^{2+}$ channel blocker TTA-P2 (*post hoc t* test, both $P$ > 0.433; fig. 7D). There were no changes in the dominant frequency of the 14 to 20-Hz band during transitions into non-REM sleep with aCSF, etomidate, or DS2 at the thalamus (all $F_{2, 16}$ < 1.95; $P$ > 0.176; fig. 7, E and F).

## Discussion

Here we show that the presence of the $GABA_A$R-targeting general anesthetic agent etomidate at the thalamus *in vivo* elicits a distinct electrocortical signature that is recapitulated by pharmacologic enhancement of phasic extrasynaptic $GABA_A$R-mediated (spillover) inhibition at the thalamus. Both the etomidate-induced and spillover-induced electroencephalogram signatures were restricted to non-REM sleep and not mediated by T-type $Ca^{2+}$ channel activation. The absence of this effect in states outside of non-REM sleep suggests that the mode of thalamic activity set up by an initial sleep transition then allows etomidate at the thalamus to enhance GABAergic spillover inhibition and elicit the distinct electrocortical signature. Increased GABAergic burst input from the RTN to thalamocortical neurons during non-REM sleep can satisfy this role.[6] In the clinical context, such a non-REM–like mode of activity would necessarily be first triggered by the sedating (*i.e.*, initial sleep promoting) properties of $GABA_A$R–targeting general anesthetic agents acting at various sites in the neuraxis.[36] The subsequent action of the general anesthetic at the thalamus would then elicit a thalamocortical network oscillation in the α-β range, a pattern that signals anesthetic hypnosis and is thought to impede responsiveness to external stimuli.[1–3] In this model, an initial sleep-like transition is a prerequisite to generate this electrocortical signature associated with anesthetic hypnosis.

Microperfusion of etomidate into the thalamus of freely behaving mice elicited an increase in non-REM α-β electrocortical activity, increased spindle-like oscillations, and decreased wakefulness. These findings with etomidate replicate previous results from our laboratory that formed the foundation for this study, and we further demonstrate that none of these effects of etomidate are sensitive to blockade of T-type $Ca^{2+}$ channels by TTA-P2.[10] Moreover, the same results with DS2 show that the electrocortical effects elicited by etomidate at the thalamus can be achieved by pharmacologic enhancement of phasic activation of thalamic extrasynaptic $GABA_A$Rs.

We previously showed that microperfusion of etomidate into the thalamus of freely behaving *Gabrd*$^{-/-}$ mice elicits alterations in electrocortical activity that mimic those identified in wild-type mice.[10] This finding shows that extrasynaptic δ$GABA_A$Rs are not necessary in mediating the electrocortical signature produced by etomidate at the thalamus. Importantly, this finding does not eliminate a role for other non-δ subunit–containing extra- or perisynaptic $GABA_A$Rs in mediating the electrocortical signature elicited by etomidate. Compensatory changes in $GABA_A$R subunit expression in *Gabrd*$^{-/-}$ mice have been reported.[37–39] Of particular relevance, *Gabrd*$^{-/-}$ mice exhibit greater coassembly of $\alpha_4$ and $\gamma_2$ $GABA_A$R subunits as indicated through immunoprecipitation, suggesting the formation of extrasynaptic $GABA_A$Rs with atypical subunit composition in *Gabrd*$^{-/-}$ mice.[37] Additionally, tonic $GABA_A$R-mediated inhibition persists in *Gabrd*$^{-/-}$ mice, albeit attenuated, indicating the presence of other functional extrasynaptic $GABA_A$Rs.[29]

The changes in IPSP duration identified *in vitro* with DS2 and etomidate require expression of extrasynaptic $GABA_A$Rs, identifying a role for spillover inhibition in mediating these changes.[7,8,24] Moreover, the effects of DS2 and etomidate on the activity of thalamocortical neurons *in vitro* are similar to each other, consistent with the *in vivo* findings with DS2 and etomidate in the present report.[7,8] Specifically, whole-cell recordings from the ventrobasal complex of the thalamus identify similar alterations in RTN-mediated IPSPs with DS2 and etomidate; both DS2 and etomidate are associated with increased IPSP decay times and charge transfer during presynaptic RTN spike bursts.[7,8,24]

The potentiation of GABA responses and alterations in IPSP duration identified with DS2 and etomidate *in vitro* satisfy the conditions required by a computational model that shows that such alterations in GABAergic thalamic signaling can precipitate the changes in cortical activity typically associated with loss of consciousness with propofol, another prototypic $GABA_A$R-targeting intravenous general anesthetic agent.[3] Importantly, the findings of our study indicate that the increase in α-β electrocortical activity observed during the induction and maintenance of anesthesia can be effectively triggered by the action of the anesthetic at the thalamus, with the stipulation that the thalamus is receiving non-REM–like inputs as the effects were restricted to non-REM sleep (figs. 2, 5, and 7).[2,3]

The findings with THIP, an agent that directly activates δ$GABA_A$Rs, and the T-type $Ca^{2+}$ channel blocker TTA-P2 indicate that alterations in thalamocortical activity that are T-type $Ca^{2+}$ channel dependent contribute to the changes in electrocortical activity identified with THIP *in vivo*. This suggestion is consistent with previous results obtained

*in vitro*.[35] Coadministration of TTA-P2 with THIP further increased slow electrocortical activity. This "potentiating" effect of TTA-P2 on slow electroencephalogram activity may reflect a diminution of thalamic activity, as THIP hyperpolarizes thalamocortical neurons to levels that can trigger a switch from tonic firing to T-type $Ca^{2+}$ channel–dependent burst firing.[35] Such inhibition of thalamocortical activity is associated with increased 1 to 4-Hz electrocortical activity and non-REM sleep *in vivo*, which is consistent with our findings with THIP and TTA-P2 at the thalamus.[40,41]

Blockade of T-type $Ca^{2+}$ channels with TTA-P2 during microperfusion of the thalamus with DS2 or etomidate did not alter any of the electrocortical effects elicited by DS2 or etomidate alone. These findings indicate that T-type $Ca^{2+}$ channel–independent alterations in thalamocortical activity underlie the changes in cortical activity identified with DS2 and etomidate. The lack of effect of TTA-P2 on the effects identified with etomidate is consistent with a previous study showing the normal onset of propofol-induced anesthesia/hypnosis in mice that lacked $Ca_v3.1$ ($\alpha^{1G}$) T-type $Ca^{2+}$ channels.[42] These channels are strongly expressed in thalamocortical relay neurons and are necessary for burst firing in these neurons.[34,43]

Both DS2 and etomidate at the thalamus elicited an increase in spindle-like oscillations, a signal that characterizes light non-REM sleep. These oscillations are generated by the thalamus and are thought to require activation of thalamocortical T-type $Ca^{2+}$ channels for their initiation and propagation.[6] Importantly, the present findings with TTA-P2 are consistent with a recent study where normal spindle density and durations were identified in mice lacking $Ca_v3.1$ channels with no thalamocortical burst firing.[44]

Coadministration of TTA-P2 with etomidate potentiated the etomidate-induced alterations in sleep–wake behavior. This finding is consistent with *in vitro* studies that identify etomidate as a blocker of T-type $Ca^{2+}$ channels.[45,46] Importantly, the concentrations of etomidate associated with T-type $Ca^{2+}$ channel blockade are significantly above the clinically relevant range ($IC_{50}$ of approximately 161 μM), and so it is unlikely that the effective concentrations of etomidate used in our study resulted in any direct effect of etomidate on thalamic T-type $Ca^{2+}$ channels. It is possible, however, that the concomitant blockade of thalamic T-type $Ca^{2+}$ channels during microperfusion of etomidate into the thalamus may have mimicked conditions of elevated etomidate concentration and facilitated the effect of etomidate on sleep–wake behavior.

While coadministration of TTA-P2 with etomidate potentiated the etomidate-induced alterations in sleep–wake behavior, TTA-P2 had the opposite effect when it was coadministered with DS2. Specifically, coadministration of TTA-P2 with DS2 restored the amount of wakefulness to levels that were comparable to baseline. Additionally, the increase in non-REM sleep with DS2 at the thalamus was attenuated with TTA-P2 (*i.e.*, the amount of non-REM sleep associated with DS2 and TTA-P2 at the thalamus was not significantly different from the amount associated with DS2 alone or at baseline). These findings suggest that the alterations in sleep–wake behavior triggered by DS2 at the thalamus may require T-type $Ca^{2+}$ channel deinactivation to occur. The molecular mechanisms underlying this differential effect of TTA-P2 on sleep–wake behavior when coadministered with etomidate *versus* DS2 are unclear and require further study. It is possible that the effect of etomidate on sleep–wake behavior is mediated through its actions on $GABA_A$Rs that are not sensitive to DS2 and do not require T-type $Ca^{2+}$ activity to influence sleep–wake behavior.

Nonetheless, given the TTA-P2–insensitive increase in α-β frequency electrocortical activity and spindle-like oscillations and the association of these respective electrocortical patterns with anesthetic-induced loss of consciousness and decreased arousal, we anticipate that mice receiving DS2 or etomidate into the thalamus would have an elevated threshold for arousal from non-REM sleep.[47–49] Future experiments that systematically assess arousal threshold in mice during such intrathalamic manipulations will confirm or refute these hypotheses and provide further insight into the clinical significance of the electrocortical signatures and alterations in sleep–wake behavior identified in our studies.

In summary, the findings in this report identify a site (thalamus) and mechanism of action (spillover inhibition) by which a prototypic $GABA_A$R-targeting general anesthetic (etomidate) can elicit the distinctive brain wave signature that accompanies anesthetic hypnosis in humans. Future studies that characterize the functional relationship between enhanced thalamic GABAergic spillover inhibition and cortical connectivity will further elucidate the neural correlates of consciousness and increase understanding of how general anesthetic agents trigger the reversible state of unconsciousness.

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## Competing Interests

The authors declare no competing interests.

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